CHEMISTRY AT LIQUID-LIQUID INTERFACES:
CON- AND COUNTER-CURRENT EXTRACTION
AND
BIOLOGICALLY ACTIVE MEMBRANES IN MICROCHANNELS

BY

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THESIS

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ABSTRACT

Surface modification of microchannels with a photo-reactive self-assembled monolayer (SAM) allows for the stable photo-patterning of liquid-air interfaces within the microchannel. It is also found that these hydrophobic/hydrophilic interfaces allow for stable interfaces between immiscible fluids. Chemistry can be conducted at these interfaces. Reversed-micelle extraction of metal ions is studied. Traditionally, fluid flow of immiscible liquids is not stable and requires controlled microchannel geometries and flow rates. The stability of the liquid-liquid interface allows for exploration in varying flow profiles within the microchannel that could not be previously studied. In particular, the flow profile in which one phase is left stationary, con- and counter-current flow. It is found that reversed-micelle extraction does not behave like typical diffusion based extraction. What is observed is an unusual perturbation at the liquid-liquid interface during the extraction that leads to turbulent flow in the microchannel. This turbulent flow differs based on the type of fluid flow in the microchannel. The ability to create stable liquid-liquid interfaces allows for the formation of membranes within the microchannel via interfacial polymerization. The stability of liquid-liquid interfaces allows for membrane formation via interfacial polymerization. Polyamide membranes are created incorporating amino acid residues. The selection of the amino acid sequence allows for the ability to create biologically active membranes that can potentially be used as biological sensors. Biologically active membranes that are reactive to chymotrypsin are synthesized and studied. It is found that these polyamide membranes can be synthesized within the microchannel and have shown reactivity toward chymotrypsin. The result of the reaction with chymotrypsin leads to a breakdown in the membranes porosity that can be visualized as a change in spontaneous fluid flow or it can lead to total dissolution of the membrane itself.
Dedicated to the man who taught me to value education
my grandfather
Charles Layugan
and
my pugs Tofu, Char-siu and Siu mai
There’s nothing like a dog’s love to pull someone out of depression
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CHAPTER 1
INTRODUCTION

1.1 MANIPULATION OF LIQUIDS IN MICROCHANNELS

Surface and liquid properties such as viscosity, adhesive and cohesive forces have little influence in large scale bulk solutions. These properties have much greater effect when the dimensions of the system begin to decrease where the surface area of the container increases relative to the volume of the solution. Capillary action for example allows liquids to flow against gravity when the diameter of the capillary tube decreases, thus allowing adhesive and cohesive forces overcome gravity to pull liquids up a capillary tube. In a microfluidic device, where channel walls fall within the μm range, these forces again become important when considering the movement of liquid through these channels. Many techniques including thermocapillary pumping,\(^1\)\(^2\) mechanical pumping\(^3\) and electro-osmotic flow\(^4\) have been used to manipulate liquids in microchannels without taking advantage of these surface and liquid properties. Capillary pumping\(^5\) and light driven motion on photo responsive surfaces\(^6\) utilize these liquid and surface properties to manipulate liquids within a microchannel.

1.2 SURFACE MODIFICATION TO CREATE STABLE AIR-WATER INTERFACES

A photo responsive self assembled monolayer (SAM) is introduced into a microchannel to allow for selective patterning of microchannel walls via photolithography.\(^7\) The SAM is
comprised of a molecule with a photoreactive 2-nitrobenzyl core initially presenting a hydrophobic perfluoronated group at the surface. Upon irradiation, the hydrophobic perfluoronated group is removed presenting a hydrophilic carboxylate group at the surface (Figure 1.1). The changes in surface characteristics can be measured by the change in advancing contact angles (118° hydrophobic perfluoronated SAM, 67° hydrophilic carboxylate SAM).

Introduction of water to the patterned microchannel leads to a spontaneous flow down the hydrophilic surface and is contained at the hydrophilic/hydrophobic interface. The stability of this air-water interface is related to the Young-Laplace equation.\(^8\)

\[
\Delta P = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right)
\]

Where \(\Delta P\) is the pressure difference, \(\gamma\) is the liquid surface free energy and \(R_1\) and \(R_2\) are the radii of curvature in the vertical and parallel directions respectively. For a long, straight stream \(R_2\) is essentially infinite. This reduces the Young-Laplace equation to

\[
\Delta P = \frac{\gamma}{R_1}
\]

Figure 1.1: Schematic of SAM formed on microchannel walls. a) During photo-reaction, a photomask is placed onto the microchannel blocking the UV light on portions of the microchannel. b) The exposed monolayer reacts with the UV light to expose the hydrophilic carboxylate surface.
R₁ is related to the advancing contact angle θₙ and the height of the microchannel, thus relating the maximum pressure to the advancing contact angle in the following manner

\[ P_{\text{max}} = \Delta P = (2\gamma/h)\sin(\theta_n - 90) \]

### 1.3 STABLE LIQUID-LIQUID INTERFACES BETWEEN IMMISCIBLE LIQUIDS

Stable liquid-liquid interfaces between immiscible liquids can also be achieved. First, the photopatterned hydrophilic section of the microchannel is filled with water. The hydrophobic section of the microchannel can then be filled with an organic solvent (Figure 1.2). The location of the liquid-liquid interface is determined by the location of the hydrophilic/hydrophobic interface achieved via photopatterning.

*Figure 1.2:* Images of microchannel. a) Unmodified microchannel containing SAM. The image on the upper right corner is the photomask used to pattern the surface. The central outlets will be exposed to the UV light converting the surface into a hydrophilic surface. b) After photopatterning, introduction of a dyed water solution to highlight the photopatterned hydrophilic surface. c and d) The hydrophobic arm is filled with a dyed toluene solution highlighting the stable liquid-liquid interface between water and toluene.
The Young-Leplace equation can be applied to the system, however the advancing contact angles now need to be adjusted to include the liquid-liquid contact between immiscible liquids where $\theta_{\text{water/org}}$ is the advancing contact angle of water surrounded by organic solvent, $\theta_{\text{org/water}}$ is the advancing contact angle of the organic phase in water. Advancing contact angles are measured for hexadecane/water systems at $143^\circ$ and $123^\circ$ respectively.

1.4 COUNTER-CURRENT LIQUID FLOW IN MICROCHANNELS

Counter-current liquid flow has been known to be the more efficient mass and heat transfer system compared to con-current flow. Nature has adopted this counter-current flow for gas exchange between air and blood in bird lungs and between water and blood in fish gills.\textsuperscript{10} Researchers have developed co- and counter-current flows of immiscible solutions in the laminar flow regime. Most of the studies involve solvent extraction.\textsuperscript{11,12} The shape of the liquid-liquid interface involving counter-current flow has been studied.\textsuperscript{13} It is found that there are two pressures that are involved in counter-current flow. The first is pressure driven flow and the second is the Laplace pressure.\textsuperscript{14} The researchers have found large deformation of the interface near the center of the microchannels. Their result also suggests spiral-like flow when under counter-current flow. This type of turbulent flow can aid in rapid solvent extraction under counter-current flow.
1.5 MEMBRANE USES IN MICROCHANNELS

Most in-situ membrane formation within the microfluidic platform has been directed toward the formation of biodegradable polymeric nanoparticles. These have attracted attention due to their high potential for site-specific drug delivery of proteins, peptides and gene therapy.\textsuperscript{15} The microfluidic platform has an advantage in the formation of very uniform nanoparticles. This uniformity is essential in the consistent dosage administered. The preparation of hydrocortisone nanoparticles in a microfluidic Y-junction has been successful.\textsuperscript{16} Solid lipid nanoparticles are also successfully prepared in microchannels.\textsuperscript{17,18} Poly(ε-caprolactone) and Poly(dl-lactic acid) nanoparticles are prepared in a coaxial glass capillary.\textsuperscript{19,20}

1.6 SCOPE AND ORGANIZATION OF THE THESIS

The research presented in this thesis will focus on the utility of the formation of stable liquid-liquid interfaces within a microchannel. With the ability to photo-pattern and control the location of the hydrophilic/hydrophobic interface stable liquid-liquid interfaces can be achieved. This allows for the exploration of liquid-liquid extraction as well as interfacial polymerization.

Chapter 2 will focus on the reversed micelle extraction of cobalt (II) into the organic phase. The chapter will present unusual turbulent flow patterns due to the formation of reversed micelles at the interface as well as differing liquid flow patterns (co- and counter-current flow).

Chapter 3 will focus on the interfacial polymerization of biologically active membranes in the microchannel. These membranes are responsive to the proteolytic enzyme chymotrypsin and will lead to a change porosity or completely dissolve when exposed to the enzyme.
1.7 REFERENCES


(7) Zhao, B.; Moore, J. S.; Beebe, D. J.; Science, 2001, 291, 1023-1026.


CHAPTER 2

FLUID BEHAVIOR OF REVERSED MICELLE EXTRACTION IN MICROCHANNELS

2.1 INTRODUCTION

Conducting chemical transformations within the microfluidic platform offers several advantages. Small amounts of reagents are used and consumed; leading to lower reagent costs and waste disposal. It is thus ideal to incorporate chemical synthesis into microfluidic systems. Unfortunately, most chemical transformations, separations and purifications often involve non-aqueous systems. Unlike liquids of similar natures, aqueous-aqueous or hexadecane-hexadecane solutions, immiscible liquids do not exhibit stable laminar interfaces. Differences in viscosity, interfacial free energies and densities lead to unstable interfaces making it difficult to control the location of these interfaces and separate the two liquids once in contact.\textsuperscript{1-3}

By patterning surface free energies within the microchannel, interesting phenomena have been observed and can be exploited to manipulate fluid flow.\textsuperscript{4,5} We have previously shown that by using self-assembled monolayers (SAMs), patterning surfaces within the microchannel can be conducted in-situ by either multi-stream laminar flow or through photolithography.\textsuperscript{6} These patterned surfaces show very stable liquid-air interfaces. It was later determined that these photopatterned surfaces also stabilizes liquid-liquid interfaces of immiscible liquids.\textsuperscript{7}

The ability to pattern stable liquid-liquid interfaces opens investigation into several chemical processes involving these interfaces. These include liquid-liquid extraction, interfacial polymerization and phase transfer reaction. In this chapter, we explore reversed micelle
extraction of cobalt (II) into hexadecane. We will investigate unusual turbulent behavior during the extraction as well as differing extraction profiles under differing flow patterns.

2.2 DETECTION OF CO(II) BY REVERSED MICELLE EXTRACTION

The detection of cobalt(II) ions is possible through reverse micelle extraction with Bis-(2-ethylhexyl) phosphoric acid (BEHPA). The hydrophobic tail makes it insoluble in water. The branched tail allows for the spontaneous self-assembly of reversed micelles in the organic phase. Extraction into the organic phase is achieved by trapping small amounts of water within the center of the reversed micelle. As the cobalt ions are extracted from the aqueous phase to the organic phase a blue color is observed indicating the successful extraction of cobalt(II).

Addition of BEHPA to the HD phase destabilizes the liquid-liquid interface within the microchannel. BEHPA is a surfactant, reducing the contact angle of HD on the modified glass surface. As a result, the liquid-liquid contact within the microchannel is not stable under static conditions, even with the added stability gained through surface modification. Stable liquid-liquid interfaces can be achieved, however, by first filling the hydrophilic half of the microchannel with aqueous cobalt (II) solution, and then pre-filling hydrophobic half of the microchannel with neat HD. The neat HD phase is then replaced by the HD containing BEHPA under applied pressure. Pre-filling the microchannel in this manner allows for stable liquid-liquid interfaces, and liquid flow through the microchannel. Both streams can be placed under fluid flow in the con-current direction. The flow of either the aqueous or the organic phase can be stopped as the other stream remains under fluid flow, all the while maintaining a stable liquid-liquid interface. The stability of the interface achieved through surface modification, allows for
the flow of liquid not only in the con-current direction, but it allows for stable flow in the counter-current direction. Counter-current flow in microchannels is typically difficult to achieve, normally requiring complex fabrication, or the introduction of membranes to physically separate the two liquid streams. The extraction of cobalt differs visually under differing flow conditions.

2.3 STATIC ORGANIC PHASE, AQUEOUS PHASE UNDER FLOW

The concentration at the liquid-liquid interface remains at a steady state because the aqueous phase is under fluid flow. Although the organic phase is under no applied pressure, the fluid will be under sheer flow due to the flowing aqueous phase. The resulting sheer flow in the organic phase is less complex than, the pressure driven flows discussed later. As the extraction begins we see very little perturbation of the liquid-liquid interface. A vortex does appear at the interface. Extraction is observed on either side of the vortex. This vortex point travels down the length of the liquid-liquid interface in the direction of fluid flow. The extraction is not symmetric about the vortex. The blue color is more diffuse on the right side of the vortex, upstream from the direction of fluid flow. The color is more intense closer to the liquid-liquid interface on the left side of the vortex. As the extraction continues over time a perturbation of the liquid-liquid interface is observed at the vortex point. It appears to pull the liquid-liquid interface toward the organic phase of the microchannel during extraction (Figure 2.1).
It is believed that the BEHPA partitions at the liquid-liquid interface. The shear stress produced by the flowing liquid disrupts the BEHPA layers at the interface forming the reversed micelles to extract cobalt into the organic phase. Extraction is believed to occur at the vortices. This results in the turbulent extraction profile.

There are two distinct flow patterns that can be used to explain the extraction profile observed. Although no pressure is applied to the organic phase, it is under motion due to the flow of the aqueous phase. The liquid flow of the organic phase can be approximated to a moving plate flow (Figure 2.2a). The maximum velocity in the organic phase is at the liquid-liquid interface. At the physical wall of the microchannel, the velocity drops down to zero. The velocity decreases linearly from the liquid-liquid interface to the microchannel wall.

Figure 2.1: a-f) Sequential images of the reversed micelle extraction of cobalt (II) ions. The aqueous phase is under applied pressure and the organic phase remains stationary. Scale bar = 200 µm
The extraction appears to initiate at the vortex perturbation points. The reverse micelles containing the cobalt (II) ions travel into the organic phase perpendicular to the liquid-liquid interface and at an angle projecting to either side of the vortex point (Figure 2.2b). Utilizing these two fluid profiles, the asymmetric profile about the vortex can be explained. To the right of the vortex, the cobalt (II) ions travel orthogonal to the liquid-liquid interface and against the flow of the organic phase. As a result, the velocity of reversed micelle in the horizontal direction begins to slow. These reversed micelles are then allowed to diffuse deeper into the organic phase. To the left of the vortex, the cobalt (II) ions again travel orthogonal to the liquid-liquid interface. However, these reversed micelles travel with the flow of the organic phase. These micelles travel faster down the length of the microchannel than they do diffusing deeper into the organic phase. Because these micelles do not travel a great distance from the liquid-liquid interface, a larger concentration of cobalt is found near the interface. These results in a more intense blue color on the left side of the vortex compared to the right side of the vortex. By understanding the fluid behavior of the liquid flows as well as the direction of the reversed micelles containing the cobalt (II) ions travel, the extraction profiles observed under different fluid flows can be elucidated.

Figure 2.2: a) Velocity profile of the system. The liquid-liquid interface is represented as the horizontal dotted line. A parabolic velocity profile is expected for the aqueous phase (lower half). Although the organic phase (upper half) is not under applied pressure it will be affected by the moving aqueous phase. b) Direction micelles travel when extracted into the organic phase. Scale bar = 200 µm
2.4 STATIC AQUEOUS PHASE, ORGANIC PHASE UNDER FLOW

The fluid profile is reversed compared to the previous one described. The aqueous phase is not under applied pressure, but does experience moving plate flow because of the liquid-liquid contact with the flowing organic phase. The organic phase is under applied pressure and will experience pressure driven parabolic flow. At the microchannel wall the velocity of the liquid approaches zero. The liquid-liquid interface is not a physical wall, as a result, the pressure driven flow of the organic phase does not approach zero at the interface. The horizontal velocity of the organic phase increases as the distance orthogonal to the liquid-liquid interface increases. As we move further away from the liquid-liquid interface, the horizontal velocity approaches a maximum. The velocity continues to decrease and approaches zero as it approaches the microchannel wall (Figure 2.3).

Diffusion-based extraction is again not observed as cobalt (II) ions are extracted into the organic phase. Laminar flow is also not observed in the organic phase. Multiple vortices are
observed throughout the length of the microchannel. These vortices are not as stable compared to the flow system discussed previously. They do not persist throughout the length of the microchannel, appear to form quickly and dissipate randomly. Micelles also appear to travel against the fluid flow of the organic phase, and then reverse direction as the micelles move away from the liquid-liquid interface. This can be attributed to the change in velocity profile of the organic phase under pressure driven flow. As a vortex point is formed, micelles to the right of the vortex travel against the flow of the organic phase, and away from the liquid-liquid interface. However, as the micelles travel further away from the liquid-liquid interface, the velocity of the organic phase increases. This results in a reversal of the direction of the micelle as it travels further away from the liquid-liquid interface (Figure 2.4a).

**Figure 2.4:** a) Direction reversed micelles travel when extracted into the organic phase. The reversal in direction of the micelles can be rationalized by the parabolic velocity profile of the organic phase under pressure-driven flow. b) Rationalization of the formation of the ball-like features commonly observed throughout the duration of the extraction. Accumulation of the reversed-micelles into these ball-like features can be attributed to close spacing between vortex points. Scale bar = 200 μm

The micelles extracted into the organic phase accumulate near the liquid-liquid interface. The higher concentrations of these micelles form amorphous ball-like structures. These features can be rationalized by the presence of two vortices being formed near each other (Figure 2.4b). The location of the two vortices can be found on either side of the ball like features. The
micelles are extracted at the vortices, travel into the hexadecane phase and begins to arch back toward the liquid-liquid interface.

The extraction of cobalt is also not continuous throughout the length of the liquid-liquid interface. Extraction of cobalt (II) ions is initially very rapid as observed by the darker blue color at the interface. The interface also is disturbed initially as the extraction starts. As the extraction continues, the intensity of the blue color fades indicating a decline in extraction. Eventually, very little extraction is observed over time. The reversed micelle extraction of cobalt is very fast and efficient, reducing the concentration of cobalt in the aqueous phase near the interface, quicker than cobalt in the aqueous phase can diffuse toward the interface. The initial disturbance of the liquid-liquid interface observed at the start of the extraction is also no longer observed. This disturbance could be attributed to the extraction process.

2.5 CONCURRENT FLOW

Under con-current flow, both the aqueous and organic phase is under applied pressure and flow in the same direction. The velocity of the fluid near the liquid-liquid interface is believed to be faster under con-current flow compared to the two previous flow profiles. This is due to the added effect of the moving plate flow caused by the contact with the adjacent fluid phase. The velocity profiles of the two streams can be approximated by adding the two profiles of the systems previously discussed where one fluid is under applied pressure and the other is not. This results in a parabolic profile in which the fluid velocity increases as it moves further away from the liquid-liquid interface. The velocity reaches a maximum velocity and begins to decrease as it approaches the microchannel wall (Figure 2.5).
Ball-like features previously observed under organic flow/static aqueous phase is again observed. The extraction rotates counterclockwise as the entire mass progresses forward in the direction of flow (Figure 2.6).

This is not a surprise as the velocity profile in the organic phase is still a parabolic profile. These features differ in that they have a long trailing tail as the mass travels in the direction of fluid.
flow. At the beginning of the extraction, we see the formation of these ball-like features. These features initially are fairly symmetric, however as the extraction continues, and the structures begin to move in the direction of fluid flow, the structure becomes asymmetric. A tail begins to form and slowly trails the core ball-like structure. The formation of these perturbation points where extraction of cobalt initially begins is random and also fleeting. It appears that more perturbation points are formed in the initial stages of the extraction. As the extraction continues however, less perturbation points are observed. This results in a non-continuous extraction throughout the length of the interface. Although there are large areas of extraction is observed, there are also long distances in which little to no extraction is observed.

2.6 COUNTER-CURRENT FLOW

The stability achieved between the immiscible fluids through surface patterning also allows for stable counter-current flow. Achieving stable counter-current flow in microchannels is not trivial and typically involves special lithography or the introduction of membranes to physically separate the two liquids. The liquid-liquid contact is achieved by surface patterning produces a large liquid-liquid contact allowing for more efficient mass transfer.

Both streams are under applied pressure. As a result, parabolic velocity profiles are again expected. There are again two factors contributing toward the velocity of the liquids; pressure driven flow, and moving plate flow. The streams travel in opposite directions complicating the velocity profiles. The velocity attributed to the pressure driven flow is counteracted by the contribution of the moving plate flow of the adjacent liquid traveling in the opposite direction. If the liquids are similar in viscosity and density, advancing contact angles and the liquid-liquid
interface is in the center of the microchannel walls, it is expected that the pressure driven flow and the moving plate flow will be the same for both liquids. The only difference would be that the two liquids are traveling in opposite directions. As a result, the velocity at the liquid-liquid interface approaches zero. The velocity profile expected is a parabolic profile that starts at zero at the microchannel wall. The velocity increases as it moves toward the liquid-liquid interface, approaches a maximum, decreases and approaches zero at the liquid-liquid interface. The direction of the liquid flow then reverses, increasing until it approaches a maximum, then decreases and approaches zero at the microchannel wall (Figure 2.7a). The two liquids are not the same in viscosity and density. The point at which the direction of the fluid flow reverses is not expected to be at the liquid-liquid interface. It is unknown what the velocity profile of the system is near the liquid-liquid interface (Figure 2.7b).

Figure 2.7: Velocity profile of counter-current flow (dotted lines). a) Ideal case in which the liquids have the same densities and viscosities, under identical applied pressures, and the liquid-liquid interface is in the center of the microchannel walls. Contribution of pressure driven flow is in opposite directions (blue arrow) is partially cancelled by the contribution due to moving plate flow (red arrows). b) The two liquids have differing densities and viscosities and the liquid-liquid interface is not in the center of the microchannel wall results in a non-ideal case in which the point where the fluid flow reverses is not at the liquid-liquid interface.

The extraction of cobalt (II) ions under counter-current flow also produces turbulent extraction profiles. However unlike the rolling ball-like extraction with the long trailing tails observed under con-current flow, vortices are formed producing a more turbulent extraction of cobalt into the organic phase (Figure 2.8).
Multiple vortices are formed throughout the length of the microchannel. These vortices are rapidly formed and transient; quickly dissipating after formation. The direction the micelles travel when initially extracted into the organic phase appear to travel in the direction of organic flow. They travel from the liquid-liquid interface deeper into the organic phase and begin to arch back toward the liquid-liquid interface. These micelles accumulate near the liquid-liquid interface, forming ball-like structures. The direction these structures travel down the microchannel, surprisingly, is not in the direction of organic flow, but against it. It appears that the contribution of the moving plate flow attributed to the aqueous phase is greater than that of the pressure driven flow of the organic phase. As a result, the point at which the liquid does not flow is not at the liquid-liquid interface, but in the organic phase (Figure 2.9).

**Figure 2.8:** a-d) Sequential optical micrographs of a reversed micelle extraction in the counter-current direction. Direction of applied pressure is indicated by the arrows. Scale bar = 200 µm
At higher flow rates the extraction is observed to be even more complex. The extraction observed initially is a very rapid extraction throughout the length of the liquid-liquid interface. The micelles near the interface appear to travel in the opposite direction of the organic phase flow. As the extraction continues more micelles are observed deeper into the organic phase. These micelles further away from the interface appear to travel in the direction of the organic phase flow. Eventually it appears that large amounts of micelles begin to travel in the direction of organic flow. It is difficult to determine if the velocity profile of the system changes during the extraction. A change in velocities would seem unlikely. What is believed to occur is that near the liquid-liquid interface the extraction travels in the direction opposite of the organic flow. As the extraction continues and more micelles travel deeper into the organic phase, the micelles encounter the point at which the fluid reverses and travels in the direction of organic flow. More
and more micelles are extracted into the organic phase increasing the concentration of cobalt. Eventually it becomes increasingly difficult to observe the extraction near the liquid-liquid interface, as most of the micelles appear to travel in the direction of the organic flow.

The counter-current extraction appears to be more efficient than the con-current flow. Extraction of cobalt (II) ions appears throughout the length of the liquid-liquid interface under counter-current flow. More turbulent flows are observed, indicating that there are more vortices forming along the interface. The more vortices forming, the higher the rate of extraction into the organic phase.

2.7 CONCLUSIONS

With the ability to photo-pattern hydrophobic and hydrophilic surfaces within the microchannel, stable liquid-liquid contact with immiscible fluids can be achieved. Pressure-driven flow not only in the con-current direction is possible, but also scenarios where one fluid is under flow and the other remains stationary, as well as counter-current flow with no perturbation of the liquid-liquid interface. Reversed micelle extraction of cobalt (II) ions into the organic phase has been successful. Unlike diffusion based extraction, these reversed micelles are extracted into the organic phase through perturbation points along the liquid-liquid interface. Traveling deeper into the organic phase, arching both to the left and right of the perturbation point and finally arching toward the liquid-liquid interface, the micelles accumulate near the liquid-liquid interface on either side of these perturbation points. The turbulent behavior of the extraction of ions differs as the pressure-driven flow conditions change.
There are two contributing velocity profiles that affect the extraction observed under differing flow conditions. The first is the parabolic velocity profile due to pressure driven flow. The second is the moving plate flow that influences the fluid phase adjacent to the phase under pressure driven flow. It is the combination of these two contributing profiles that changes the reversed micelle extraction observed when the fluids are under differing flow conditions. When the aqueous phase is under applied pressure and the organic phase is remains stationary, the perturbation points are clearly visible.

The extraction of cobalt (II) ions under con-current flow is not as efficient as counter-current flow. No extraction is observed for long distances along the liquid-liquid interface under con-current flow. Under counter-current flow, multiple vortices are observed throughout the length of the channel.

2.8 EXPERIMENTAL SECTION

Materials

Hexadecane, Bis-(2-ethylhexyl) phosphoric acid (BEHPA), Co(OAc$_2$) was purchased from Sigma. All reagents were used without further purification. Glass microscope slides (75 x 50 x 1 mm) and cover slips (22 x 50 mm, No. 1 thickness, 180 µm thickness) were purchased from Fischer Scientific. UV-curable adhesive (Norland Optical Adhesive No 61) was purchased from Norland Products (New Brunswick, NJ). Photomasks were prepared in Adobe Illustrator and printed on transparency film with a high-resolution printer (5080 dpi, Linotype Herkules Imagesetter, Heidelberg, Germany).
Instrumentation

Polymerization and characterization were performed on an Olympus Epi-Fluorescent microscope (BX-60) equipped with a Sony CCD-IRIS/RGB color video camera and a Panasonic AG-1980 videocassette recorder with monitor. The light source was filtered through a near UV filter cube with a 360-370 nm band-pass (U-MNUA, type BP360-370). A Harvard Apparatus PHD Programmable syringe pump was used for the enzymatic digestion of the peptide membranes.

Synthesis of 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(11-trichlorosilyl-1-oxoundecyloxymethyl)-3-nitrobenzoate [3].

Synthesis of 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(11-trichlorosilyl-1-oxoundecyloxymethyl)-3-nitrobenzoate [3] involved a 3 step route as illustrated in Scheme 2.1.
2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-bromomethyl-3-nitrobenzoate [1].

4-(bromomethyl)-3-nitrobenzoic acid (2.50 g, 9.61 mmol) and 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octanol (5.77 g, 14.42 mmol) was dissolved in CH$_2$Cl$_2$ (70 mL). Once all solids were dissolved DCC (3.00 g, 14.54 mmol) and DMAP (0.12 g, 0.96 mmol) were added. The mixture was allowed to stir overnight. The precipitant was filtered off and the filtrate concentrated on a rotary evaporator. Purification was achieved via silica gel column chromatography (40% vol CH$_2$Cl$_2$ in petroleum ether). Removal of the solvent was achieved with a rotary evaporator affording a white solid (4.25 g, 6.6 mmol, 68.9%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ (ppm) 8.70 (d, 1H, aromatic), 8.26 (dd, 1H, aromatic), 7.76 (d, 1H, aromatic), 4.88 (t, 2H, -CH$_2$CF$_2$-), 4.84 (s, 2H, -CH$_2$Br). $^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ (ppm) 162.73, 148.36, 138.45, 134.58, 133.41, 130.01, 127.16, 60.83 (t, 60.05, 60.61), 27.84. MS (FD) 642.8 (100%).

2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(1-oxo-10-undecenyloxymethyl)-3-nitrobenzoate [2].

NaOH (0.68 g, 17.00 mmol) and undecylenic acid (2.11 g, 11.45 mmol) was dissolved in DI H$_2$O (2.60 mL) and HMPA (30 mL). The mixture was allowed to stir at room temperature for 2 h. The nitrobenzoate [1] (3.65 g, 5.68 mmol) dissolved in HMPA (20 mL) was added dropwise to the stirring solution. The solution was allowed to stir at room temperature overnight and poured onto 0.1 M HCl solution (200 mL). The pH value was adjusted to 7 with 2 M NaOH. The solution was extracted with ether (4 x 50 mL). The organic layers were combined and dried over anhydrous MgSO$_4$. The solvent was removed using a rotary evaporator and the crude product purified by silica gel column chromatography (50% vol CH$_2$Cl$_2$ in petroleum ether).
Removal of the solvent was achieved with a rotary evaporator to afford a white waxy solid (3.00g, 4.1 mmol, 62.0%). $^1$H NMR (500 MHz, CDCl$_3$): δ (ppm) 8.74 (d, 1H, aromatic), 8.31 (dd, 1H, aromatic), 7.75 (d, 1H, aromatic), 5.81 (m, 1H), 5.56 (s, 2H, -CH$_2$O), 5.01-4.85 (m, 4H), 2.44 (t, 2H), 2.04 (m, 2H), 1.66 (m, 2H), 1.38-1.25 (m, 10H). $^{13}$C NMR (500 MHz, CDCl$_3$): δ (ppm) 173.17, 162.93, 147.77, 139.36, 138.46, 134.62, 129.52, 129.27, 126.76, 114.37, 62.61, 60.76 (t, 61.00, 60.57), 34.28, 33.87, 29.47, 29.35, 29.23, 29.06, 25.04. MS (FD) 745.2 (100%).

2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(11-trichlorosilyl-1-oxoundecyloxymethyl)-3-nitrobenzoate [3].

The alkene [2] (0.60 g, 0.81 mmol) was added to a round bottomed flask attached to a schlenk line. Air was removed and replaced with N$_2$. The alkene was dissolved in dry CH$_2$Cl$_2$ (2 mL) and trichlorosilane (4.0 mL) was added. A solution of hydrogen hexachloroplatinate (IV) hydrate (20 µL, 15.6 mg) in 2-propanol (0.5 mL) was injected into the flask via micro syringe. The solution was heated to reflux and allowed to react overnight. Removal of the solvent was achieved via reduced pressure on a schlenk line to afford a waxy solid. The product was used to form SAMs on glass surfaces without further purification. $^1$H NMR (500 MHz, CDCl$_3$): δ (ppm) 8.74 (d, 1H, aromatic), 8.31 (dd, 1H, aromatic), 7.75 (d, 1H, aromatic), 5.56 (s, 2H, -CH$_2$OOC-), 4.89 (t, 2H, -OCH$_2$CF$_2$), 2.44 (t, 2H), 1.66 (m, 2H), 1.56 (m, 2H), 1.29 (m, 14H). $^{13}$C NMR (500 MHz, CDCl$_3$): δ (ppm) 173.17, 162.93, 147.81, 138.40, 134.62, 129.52, 129.31, 126.76, 62.61, 60.76, 34.28, 31.98, 29.56, 29.45, 29.37, 29.30, 29.17, 25.03, 24.46, 22.44.
Microchannel Formation

All microscope slides and cover slips were first cleaned with a freshly prepared “piranha” solution (70:30 v/v conc H$_2$SO$_4$:H$_2$O$_2$) at 60 °C for 30 min, rinsed with DI H$_2$O and dried with a stream of clean air. Microchannels were made by affixing No. 1 glass cover slips onto a glass microscope slide (Fisher Scientific) with a UV-curable glue in such a way that the cover slips form the side walls of an H-channel. The glue was cured for 5 min with a Kinsten Exposure Unit (KVP-30, Computronic Corp LTD, Australia). The process was repeated with a larger coverslip to form the top wall of the microchannel. The resulting microchannels were approximately 190 µm deep. Plastic micropipette tips were trimmed to a length of 0.5 in and attached to the opening of the microchannel with DURO Quick Set epoxy resin to form the inlet and outlet wells of the microchannel.

FSAM formation and Photopatterning

A solution is prepared by dissolving the trichlorosilyl [3] (22 mg) in toluene (8.0 g) in a scintillation vial. The solution was allowed to age for a certain period of time until 1 min immersion of a “piranha” treated coverslip resulted in a H$_2$O advancing contact angle of 118°. Once optimal aging has been achieved the microchannels are filled with this solution and allowed to stand for 1 min. After exposure, the microchannels are rinsed with hexane, followed by methanol and allowed to dry with a clean stream of air. If the aging solution becomes cloudy, the solution is filtered with a 0.45 µm PTFE syringe filter and the aging process is repeated until a contact angle of 118° is again achieved.

Photopatterning of the FSAM was achieved by filling the microchannels with a 0.1 M HCl solution. A photomask is placed to cover one half of the H-channel and a cover slip was
placed onto the photomask to ensure that it lies flat. The UV intensity of Novacure systems (EFOS, Model N2001-A1) was preset at 5600 mW/cm$^2$, the distance between the lens and the channel was 40 mm, and the irradiation area was 20 mm in diameter. The microchannel was irradiated for 6 min. The inlet and outlet ports were also irradiated to create the hydrophilic arms of the H-channel. The microchannels were rinsed with DI H$_2$O, followed by methanol and dried with a stream of clean air. A sample coverslip was also irradiated in the same manner to measure the advancing contact angle. UV-irradiated FSAM decreased from $118^\circ$ to $67^\circ$.

**Reversed Micelle Extraction of Co(II)**

An aqueous solution of Co(Ac)$_2$ (0.03 M) is prepared and the pH adjusted to 5.8 with acetic acid. A 0.1 M solution of Bis-(2-ethylhexyl) phosphoric acid (BEHPA) is prepared in hexadecane (HD). In a pre-patterned microchannel, the hydrophilic portion of the microchannel is filled with the aqueous Co(Ac)$_2$ solution. Pure HD is then used to fill the hydrophobic half of the microchannel. The HD is then displaced with the BEHPA in HD utilizing a syringe pump (flow rate = 0.03 mL/min). The cobalt solution is also placed under flow via syringe pump (flow rate = 0.11 mL/min). Con and counter-current flow is achieved by location of the inlet ports on the H-channel. Visualization of the extraction is observed through a microscope.

### 2.9 REFERENCES


(3) Zhao, B.; Moore, J.S.; Beebe, D.J. Unpublished results.


CHAPTER 3

BIOLOGICALLY ACTIVE MEMBRANES IN MICROCHANNELS

3.1 INTRODUCTION

Botulinum toxin is a neurotoxic protein produced by the bacterium Clostridium Botulinum. The toxin is post-translationally proteolyzed to form a di-chain molecule linked by a disulfide bond. Once internalized the light chain disassociates, it specifically cleaves one of three different SNARE proteins essential for synaptic vesicle fusion. This cleavage results in inhibition of acetylcholine secretion ultimately leading to paralysis\textsuperscript{1,2}. It is this specific cleaving process that can provide a method of detection. If a system can be devised that allows for a visual detection of the cleaving process, a chemical sensor can be envisioned.

Microfluidics is an ideal platform as chemical sensors due to their small scales. Low concentrations of agents can be detected, as well as low amounts of reagents consumed. The lower dimensions used in microfluidics also allows the systems to become portable, leading possible data acquisition, analysis and detection on site. This can result in decreased time between exposure and detection.

Hydrogel polymers with peptide crosslinks have been found to be protease-responsive, leading to degradation of the hydrogel when the peptide crosslinks are cleaved by the protease\textsuperscript{3,4}. Membranes can be utilized as an alternative to these hydrogel polymers. Membranes acting as biological sensors possess several advantages within the microfluidic platform. Small amounts of reagents are needed to produce these membranes. Membranes are thin, lowering the diffusion path length of the agent to be detected (i.e. large enzymes, toxins and proteases) compared to
hydrogel matrices. Nylon-6,6 has previously been created in a microfluidics channel.\textsuperscript{5} If one of the monomeric groups is comprised of peptides, a biologically active polymer can be envisioned.

In this chapter, membranes incorporating short peptide sequences into the polymer chain are described. These membranes have been found to specifically degrade when exposed to chymotrypsin, either producing a change in membrane porosity or complete dissolution. This provides a visual output to the detection of enzymatic activity. Arrays of membranes have been achieved by incorporating multiple membranes within the microchannel.

### 3.2 PEPTIDE SEQUENCES

Chymotrypsin is a proteolytic enzyme that selectively cleaves on the C-side of hydrophobic residues (Phe, Tyr, and Trp). Due to its residue specificity, chymotrypsin is chosen as the model enzyme for this project. Several peptide sequences have been selected for the study with chymotrypsin. Ac-Lys-βAla-Tyr-Leu-βAla-Lys-NH\textsubscript{2} [1] is the initial peptide sequence selected. The placement of the tyrosine residue is centralized in the sequence. The amine functional group on the side chain of lysine will be able to react with adiptyl chloride to form the polyamide polymer. β-alanine residues are selected as spacer residues to allow for better interaction of tyrosine with the active hydrophobic pocket of Chymotrypsin. All peptides have been synthesized using a peptide synthesizer, deblocked from the solid phase resin, analyzed for purity via HPLC and masses confirmed using ESI mass spectroscopy. Once purity and masses have been confirmed, enzymatic degradation experiments are conducted (Table 3.1).
### Table 3.1: Peptide sequences synthesized and studied.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>LR-MS (ESI)</th>
<th>Purity (HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Lys-βAla-Tyr-Leu-βAla-Lys-NH₂ [1]</td>
<td>733.5 calc, 734.4 measured</td>
<td>96.3%</td>
</tr>
<tr>
<td>Ac-Lys-Tyr-Leu-Lys-NH₂ [2]</td>
<td>591.4 calc, 592.4 measured</td>
<td>97.8%</td>
</tr>
<tr>
<td>Ac-Lys-βAla-Gly-Leu-βAla-Lys-NH₂ [3]</td>
<td>627.8 calc, 628.4 measured</td>
<td>95.7%</td>
</tr>
<tr>
<td>Ac-Lys-βAla-Phe-Leu-βAla-Lys-NH₂ [4]</td>
<td>717.9 calc, 718.4 measured</td>
<td>97.2%</td>
</tr>
<tr>
<td>Ac-Lys-Tyr-Asn-Lys-NH₂ [5]</td>
<td>591.7 calc, 593.4 measured</td>
<td>98.3%</td>
</tr>
</tbody>
</table>

3.3 ENZYMATIC DEGRADATION OF PEPTIDE SEQUENCES

Enzymatic degradation of the peptide sequence [1] is conducted and monitored via HPLC. The peptide is observed to elute off of the HPLC column at 14.8 minutes. The peptide is exposed to the chymotrypsin solution, vortexed for one minute and left to stand. The solution is not agitated to mimic the static conditions the peptide membranes would experience in the microchannel. HPLC samples are obtained at 1h, 2h, 3h and 17h. Cleavage of the peptide sequence is observed after 1 hour (elution time 7.6 minutes). After 3 hours a significant amount of the peptide sequence remains undigested by chymotrypsin. The solution is allowed to stand at room temperature overnight and an HPLC sample had been obtained at 17 hours. At that time, the peptide is completely digested by chymotrypsin (Figure 3.1). HPLC-MS confirms the mass of the digested fragment cleaving at the C-side of tyrosine.
Figure 3.1: Enzymatic degradation of Ac-Lys-βAla-Tyr-Leu-βAla-Lys-NH$_2$ [1] with Chymotrypsin. The initial HPLC of the peptide is the bottom spectra (elution of the peptide at 14.8 minutes). Spectra sequentially moving up are after 1h, 3h and 17h of chymotrypsin exposure. Elution of the cleaved shorter peptide observed at 7.5 minutes. The peptide is observed to have been completely cleaved by chymotrypsin at 17 hours.

Several control experiments are completed. A control sequence Ac-Lys-βAla-Gly-Leu-βAla-Lys-NH$_2$ [3] is also synthesized. Enzymatic degradation experiments with chymotrypsin indicate no reaction over a period of 17 hours. The peptide sequence [1] was also exposed to the same chymotrypsin solution but containing a chymotrypsin inhibitor. No cleavage products are observed with chymotrypsin and the inhibitor.

Part of the attraction of utilizing membranes in microchannels is the idea of rapid detection. The time frame of the digestion of peptide [1] may be too long as a practical method of detection. A second, shorter peptide sequence is synthesized by removing the βAla spacer peptides (Ac-Lys-Tyr-Leu-Lys-NH$_2$ [2]). Enzymatic digestion experiments were repeated on the shorter peptide sequence [2] and monitored via HPLC. The peptide sequence elutes off of the column at 15.0 minutes. Both the longer peptide sequence [1] and the shorter peptide sequence [2] have very similar retention times. It would be expected that the digested fragment would also
have a similar retention time compared to the digested fragment from peptide [1]. The retention time for the digested fragment is observed to be the same at 7.5 minutes. The shorter peptide is also observed to be a more active substrate for the chymotrypsin enzyme. Complete digestion is observed at 3 hours (Figure 3.2).

![Figure 3.2: Chymotrypsin digestion of Ac-Lys-Tyr-Leu-Lys-NH$_2$ [2]. Retention time observed for [2] is 15.0 minutes. HPLC prior to chymotrypsin exposure is the bottom spectra. Spectra, in ascending order, is after 1h, 2h and 3h exposure to the chymotrypsin enzyme. The retention time observed for the digested product is 7.5 minutes.](image)

The peptide sequence Ac-Lys-Tyr-Asn-Lys-NH$_2$ [5] is also subject to the same enzymatic degradation experiment. Peptide [5] is similar to that of peptide [2] with the Leu residue replaced with Asn. Digestion with chymotrypsin is complete within an hour. This substrate is observed to be more active than that of peptide sequence [2].
3.4 Ac-Lys-βAla-Tyr-Leu-βAla-Lys-NH₂ MEMBRANES

Initial experiments to create stable polymers outside of the microchannel have been unsuccessful. Small fragments of polymer have been observed, but a continuous membrane has not. The observation of smaller polymerized material is optimistic. Although the reaction to form a continuous polyamide membrane is not observable in larger scales, the smaller dimensions within the microchannel may allow for the formation of stable membranes. Experiments involving peptide concentrations have been completed and stable membranes within the microchannel have been successfully prepared. Continuous membranes are formed with polymerization times of 5, 10, 15 and 30 minutes with a 0.19 M concentration of peptide, however, the 5 and 10 minute membranes were not stable after drying.

Successful polymerization leading to membrane formation is characterized in the following manner: 1) visual detection of a continuous membrane after rinsing of the microchannel, and 2) the membrane is not porous enough for methanol to diffuse through (the membrane essentially acts as a physical wall preventing solvent permeation). Stable membranes are prepared in the microchannel with a polymerization time of 15 min. The reaction is exothermic as air bubbles evolved at the liquid-liquid interface. During the polymerization a precipitate is observed diffusing into the toluene phase of the system. This can be attributed to the hydrolysis of the adipoyl chloride resulting in diacid formation, the product of which is not soluble in toluene. Despite the presence of air bubbles and a competing side reaction, continuous and stable membranes are formed after rinsing and drying. These membranes are also successful at containing methanol when one half of the microchannel is filled (Figure 3.3).
3.5 ENZYMATIC DEGRADATION OF Ac-Lys-βAla-Tyr-Leu-βAla-Lys-NH₂ MEMBRANES

The dried membranes are then exposed to the chymotrypsin solution (0.23 mg/mL pH 7.8 phosphate buffer). The entire microchannel is filled and placed in an oven (37 °C) for 24 h. A control microchannel is filled with buffer solution and placed in the oven. Upon exposure to chymotrypsin, the peptide backbone of the polymer will be cleaved by the enzyme, producing water soluble smaller fragments, leading to eventual dissolution of the membrane. After 24 h both membranes are rinsed and dried. Unfortunately, a membrane is observed for the enzyme-exposed system. The two systems cannot be distinguished visually. However differences in the fluid behavior of the two systems are observed during the rinsing of the microchannels.

In a pre-patterned microchannel, methanol is added to the hydrophobic side. The contact angle of methanol on the hydrophobic surface is <90°, thus allowing for spontaneous flow on the hydrophobic half of the channel. However, the contact angle of methanol on the hydrophilic surface is considerably lower, resulting in a greater affinity to wet the hydrophilic surface. As a result, the methanol will first spontaneously flow down the hydrophobic arm, until it encounters
the hydrophobic/hydrophilic interface. At that point, methanol will preferentially and spontaneously fill the hydrophilic surface. Once the hydrophilic surface is completely wetted, the hydrophobic surface will be wetted by methanol (Figure 3.4).

Figure 3.4: a-f) Fluid flow of methanol through the photopatterned microchannel with no membrane. The upper half of the H-channel has a hydrophobic surface, the hydrophilic surface is the lower half of the H-channel. Methanol is injected through the upper left corner of the microchannel. Methanol is dyed with methylene blue for visualization. Scale bar = 10mm

This spontaneous behavior of methanol is perturbed by the incorporation of the peptide membrane. Filling the channel with methanol from the hydrophobic arm, results in the spontaneous flow. Once it encounters the membrane, the methanol solution can either permeate through the membrane, spontaneously filling the hydrophilic surface of the microchannel, or be blocked by the membrane filling the hydrophobic half of the H-channel. What is observed is the filling of the hydrophobic pattern of the microchannel. The membrane effectively acts as a wall, preventing permeation and the spontaneous filling of the hydrophilic surface (Figure 3.5).

The same visualization technique is applied to the microchannels exposed to buffer solution and the buffered chymotrypsin solution. The microchannel exposed to buffer solution behaves in the same fashion as a channel containing a non-exposed peptide membrane; indicating no change in the membrane microstructure during incubation.
Membranes exposed to the chymotrypsin solution behave differently. Methanol is first introduced into the hydrophobic arm of the H-channel, spontaneously filling that arm. As the solution encounters the membrane, it is initially directed toward the hydrophobic side of the channel. Before completely filling the hydrophobic side of the channel, however, the methanol solution permeates through the membrane and begins to spontaneously fill the hydrophilic half of the channel. This results in the filling of both halves of the H-channel. It is believed that exposure to chymotrypsin leads to incomplete digestion of the membrane, not resulting in dissolution as initially expected, but leading to a change in membrane porosity. This microscopic change in the membrane microstructure can be visibly detected utilizing the methanolic solution. Thus it is possible to detect enzyme exposure through a change in membrane porosity (Figure 3.6).
Figure 3.6: a-d) Fluid flow of methanol through the photopatterned microchannel with membrane exposed to cymotrypsin solution. The upper half of the H-channel has a hydrophobic surface, the hydrophilic surface is the lower half of the H-channel. Methanol is injected through the upper left corner of the microchannel. Methanol is dyed with methylene blue for visualization. Scale bar = 10mm

It would, however, be more ideal to simplify the output detection to complete dissolution of the membrane. Exposure to chymotrypsin for 2 days at 37 °C results in no complete dissolution of the membrane. It is believed that increasing the concentration of enzyme in the solution would lead to membrane dissolution, but has not been investigated. Increasing the concentration of enzyme would not be a practical solution as most agents are quite potent at very dilute concentrations.

3.6 Ac-Lys-Tyr-Leu-Lys-NH2 MEMBRANES

Removal of the βAla units produces a shorter peptide sequence. HPLC enzyme degradation studies shows that this shorter sequence is more effectively cleaved by chymotrypsin compared to the first system investigated. It is, however, unknown if this trend will follow once a polymer is constructed. Stable and continuous membranes are constructed utilizing this sequence after 15 min. Prior to exposure to chymotrypsin, its behavior when exposed to methanol is also the same as the previous system. Thus we expect to see a change in porosity when exposed to chymotrypsin. After exposure to chymotrypsin and incubation at 37 °C for 24 h, no membrane was observed. Removal of the chymotrypsin solution, followed by methanol rinsing and drying results in an empty channel. The membrane is completely digested into water
soluble fragments by chymotrypsin. This simplifies the positive visual detection of the system. Exposure of the membranes to buffer solution containing no enzyme produced no change in the membrane. Membranes can also be digested at room temperature, further simplifying the sample processing (Figure 3.7).

![Figure 3.7: a) Dried peptide membrane before exposure to chymotrypsin b) Membrane exposed to buffer solution. The membrane directs the flow of methanol indicating no change in membrane microstructure when exposed to buffer solution. c) Membrane exposed to chymotrypsin. After rinsing, no membrane is observed indicating complete dissolution of the membrane when exposed to chymotrypsin. Scale bar = 400 µm](image)

Digestion of the membrane under fluid flow provides several advantages to the static approach previously utilized. Improved mass transfer of the enzyme to the membrane can be achieved, as well as improved dissolution of the water soluble fragments. Both sides of the channel are pre-filled with enzyme solution. The hydrophilic half of the channel is left static, while the solution in the hydrophobic half is cycled forward and backwards continuously (infuse 600µL/h for 4 min; withdraw 600µL/h for 4 min). This asymmetric fluid flow offers a third advantage to the system. As the membrane integrity becomes weaker during the digestion, the fluid flow is able to stress the membrane. It is then possible to mechanically break the membrane via fluid flow before total enzyme digestion is achieved, thus shortening the time for complete membrane dissolution. The stress to the membrane is observed during the course of the digestion. Early in the digestion, the membrane integrity remains high. As a result, the membrane does not shift during the liquid flow. As the membrane weakens and its adhesion to
the glass surface fails, the membrane begins to bow under the fluid flow. Eventually the membrane ruptures by the flowing liquid.

### 3.7 MEMBRANE SENSITIVITY TO CHYMOTRYPSIN

The sensitivity of the system can be addressed by decreasing the concentration of chymotrypsin. Under flowing conditions membranes are dissolved within 24 h at a concentration of 0.115 mg/mL. Concentrations as low as 0.023 mg/mL is found to also digest the membrane, however, the 1 week time required is quite long. It has been shown previously that a change in membrane porosity can be detected by observing how the membrane behaves as it is filled with methanol. This change in porosity precedes dissolution so it may be possible to increase the sensitivity of the system by detecting a change in porosity rather than total dissolution. Exposing the membranes to 0.23 mg/mL chymotrypsin becomes porous after 2 h, and the ten-fold dilution 0.023 mg/mL becomes porous after 24 h. This is a great improvement compared to the 7 days needed to completely digest the membrane. With the two detection modes possible for these microchannels, the system can serve as both an early detection and a confirmation system. The porosity change can be observed quickly and at low concentrations. Once a change in porosity is initially observed, indicating exposure to the enzyme, the channel can be filled with a higher concentration of enzyme and be dissolved within 24 h to confirm enzyme exposure (Table 3.2).
Table 3.2: Peptide membranes exposed to differing solutions and checked for changes in porosity or complete dissolution

<table>
<thead>
<tr>
<th>Membrane Exposure</th>
<th>Concentration (mg/mL)</th>
<th>MeOH Permeable</th>
<th>Membrane Dissolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td>No – 3 days</td>
<td>No – 3 days</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0.23</td>
<td>Yes – 2 h</td>
<td>Yes – 12 h</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0.115</td>
<td>Yes – 4 h</td>
<td>Yes – 24 h</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0.023</td>
<td>Yes – 24 h</td>
<td>Yes – 7 days</td>
</tr>
<tr>
<td>Chymotrypsin + Inhibitor</td>
<td>0.23</td>
<td>No – 3 days</td>
<td>No – 24 h</td>
</tr>
</tbody>
</table>

Two control experiments were conducted to determine if the change in porosity and membrane dissolution was either attributed to the fluid flow or to the enzyme. Exposure to buffer solution containing no enzyme produced no change in membrane porosity and did not lead to membrane dissolution after 3 days. Membranes exposed to a solution containing chymotrypsin and a chymotrypsin inhibitor also did not produce a change in porosity or dissolution after 24 h. Thus the change in membrane porosity and dissolution can only be attributed to specific enzyme cleavage of the peptide backbone of the membrane.

3.8 MEMBRANE ARRAYS

With the ability to pattern surfaces within the microchannel with reasonable control, creation of multiple membranes within the same microchannel is possible. Arrays of membranes can be envisioned in which the detection of multiple agents as well as mixtures can be detected. Two membranes of differing peptide sequences are incorporated into the microchannel. These membranes will be specifically active to either agent A, agent B or a mixture of the two. A model system is constructed containing two membranes, nylon 6-6, and the Ac-Lys-Tyr-Leu-Lys-NH$_2$ membrane. Nylon 6-6 contains no peptide sequence and should result in no change in
membrane integrity when exposed to chymotrypsin. The second membrane contains a peptide sequence that can be digested with chymotrypsin. Exposure of the array to buffer solution results in no change in membrane integrity of either of the membranes. This is visualized by the directed flow of MeOH through the center of the array. The flow of MeOH differs through an array exposed to chymotrypsin. As expected, exposure to chymotrypsin results in selective digestion of the peptide membrane over the nylon 6-6 membrane. As the array is exposed to MeOH, the solution permeates through the peptide membrane; filling the side of the array the peptide membrane was located. The nylon 6-6 membrane remains unaffected, preventing permeation though that membrane (Figure 3.8). With careful choice of the peptide sequence arrays can be made to detect multiple agents as well as mixtures of agents.

3.9 CONCLUSIONS

Incorporation of enzymatically active peptides can be achieved through interfacial polymerization. Membranes produced in this manner have been incorporated into the microfluidic platform. Enzymatic digestion leads to first a change in membrane porosity
followed by complete dissolution of the membrane. Both changes in the membrane structure can be observed visually (methanol flow or complete dissolution), thus removing complex detection methods such as fluorescence microscopy or electronic measurements. The sensitivity of these systems can be quite low, currently achieving detection at the µg/mL concentration range. It can be envisioned that given selection of ideal peptide sequences, more complex proteolytic systems can be detected. Arrays can be constructed by incorporating more than one membrane within the microchannel. Multiple detection of agents, as well as mixtures of agents can also be detected by these membrane arrays.

3.10 EXPERIMENTAL SECTION

Materials

All Fmoc protected amino acids, HBTU, HOBT and MBHA resin were purchased from ChemImpex. All other reagents and consumables used for peptide synthesis were purchased from Applied Biosystems. Piperidine, ethanedithiol, thioanisole, dithiothreitol, α-chymotrypsin was purchased from Sigma. All reagents were used without further purification. Glass microscope slides (75 x 50 x 1 mm) and cover slips (22 x 50 mm, No. 1 thickness, 180 μm thickness) were purchased from Fischer Scientific. UV-curable adhesive (Norland Optical Adhesive No 61) was purchased from Norland Products (New Brunswick, NJ). Photomasks were prepared in Adobe Illustrator and printed on transparency film with a high-resolution printer (5080 dpi, Linotype Herkules Imagesetter, Heidelberg, Germany).

Instrumentation
Polymerization and characterization were performed on an Olympus Epi-Fluorescent microscope (BX-60) equipped with a Sony CCD-IRIS/RGB color video camera and a Panasonic AG-1980 videocassette recorder with monitor. The light source was filtered through a near UV filter cube with a 360-370 nm band-pass (U-MNUA, type BP360-370). A Harvard Apparatus PHD Programmable syringe pump was used for the enzymatic digestion of the peptide membranes. Solid-phase peptide synthesis was accomplished on an Applied Biosystems 433A Peptide Synthesizer. Reversed phase HPLC analysis of peptides was obtained on a Waters 600 HPLC system and detected using a Waters 2487 dual wavelength detector. Analytical separation was obtained via a Vydac 218TP54 C18 reversed phase column.

**Peptides Sequences**

All peptides were synthesized on solid phase with a 433A Applied Biosystems Peptide Synthesizer, on a 0.25 mmol scale with 4 x excess of amino acid, using standard Fmoc/HBTU/HOBt chemistry on MBHA resin. Deblocking of the amino acids was accomplished through 4 h of vortexing in a solution containing trifluoroacetic acid (4 mL), phenol (0.3 g), ethanedithiol (200 µL), thioanisole (100 µL) and Millipore water (200 µL). The resin was separated via filtration and the filtrate was added dropwise to cold ether (30 mL) to precipitate the peptide. The mixture was centrifuged and decanted. The precipitation procedure was repeated three times. The peptide was then dissolved in 4 mL of 1:1 acetonitrile:Millipore water solution and lyophilized. HPLC analysis is as follows: solvent A (H₂O with 0.01% TFA), solvent B (80:20 MeCN:H₂O with 0.08% TFA). All peptides were analyzed starting at 90% solvent A:10% solvent B ramped to 40% solvent A:60% solvent B over a period of 30 min. The solvent system was then ramped to 100% B over the next 30 min, with a flow rate of 1 mL/min.
Signals were detected at 214 nm. Ac-Lys-βAla-Tyr-Leu-βAla-Lys-NH₂ [1]: LR-MS (ESI) calcd. = 733.5; actual = 734.4, 96.3% purity HPLC. Ac-Lys-Tyr-Leu-Lys-NH₂ [2]: LR-MS (ESI) calcd. = 591.4; actual = 592.4, 97.8% purity HPLC. Ac-Lys-βAla-Gly-Leu-βAla-Lys-NH₂ [3]: LR-MS (ESI) calcd. = 627.8; actual = 628.4, 95.7% purity HPLC. Ac-Lys-βAla-Phe-Leu-βAla-Lys-NH₂ [4]: LR-MS (ESI) calcd. = 717.9; actual = 718.4, 97.2% purity HPLC. Ac-Lys-Tyr-Asn-Lys-NH₂ [5]: LR-MS (ESI) calcd. = 591.7; actual = 593.4, 98.3% purity HPLC.

**Chymotrypsin digestion**

All enzymatic digestion experiments were carried out in the same manner for differing peptide sequences. A master solution of chymotrypsin in 7.8 phosphate buffer (10 mM) was prepared at a concentration of 0.23 mg/mL phosphate buffer. Peptide solutions were prepared at a concentration of 1.4-1.5 mg peptide/mL phosphate buffer. 200 µL of the chymotrypsin solution was added to the peptide solution, vortexed for 1 min and allowed to stand throughout the digestion. A 20 µL sample was removed at varying times and analyzed via Reversed Phase HPLC (using the same HPLC method).

**Synthesis of 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(11-trichlorosilyl-1-oxoundecyloxymethyl)-3-nitrobenzoate [3].**

Synthesis of 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(11-trichlorosilyl-1-oxoundecyloxymethyl)-3-nitrobenzoate [3] involved a 3 step route as illustrated in Scheme 2.1.
2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-bromomethyl-3-nitrobenzoate [1].

4-(bromomethyl)-3-nitrobenzoic acid (2.50 g, 9.61 mmol) and 2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octanol (5.77 g, 14.42 mmol) was dissolved in CH$_2$Cl$_2$ (70 mL). Once all solids were dissolved DCC (3.00 g, 14.54 mmol) and DMAP (0.12 g, 0.96 mmol) were added. The mixture was allowed to stir overnight. The precipitant was filtered off and the filtrate concentrated on a rotary evaporator. Purification was achieved via silica gel column chromatography (40% vol CH$_2$Cl$_2$ in petroleum ether). Removal of the solvent was achieved with a rotary evaporator affording a white solid (4.25 g, 6.6 mmol, 68.9%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ (ppm) 8.70 (d, 1H, aromatic), 8.26 (dd, 1H, aromatic), 7.76 (d, 1H, aromatic), 4.88 (t, 2H, -CH$_2$CF$_2$-), 4.84 (s, 2H, -CH$_2$Br). $^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ (ppm)
2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(1-oxo-10-undecenyloxymethyl)-3-nitrobenzoate [2].

NaOH (0.68 g, 17.00 mmol) and undecylenic acid (2.11 g, 11.45 mmol) was dissolved in DI H₂O (2.60 mL) and HMPA (30 mL). The mixture was allowed to stir at room temperature for 2 h. The nitrobenzoate [1] (3.65 g, 5.68 mmol) dissolved in HMPA (20 mL) was added dropwise to the stirring solution. The solution was allowed to stir at room temperature overnight and poured onto 0.1 M HCl solution (200 mL). The pH value was adjusted to 7 with 2 M NaOH. The solution was extracted with ether (4 x 50 mL). The organic layers were combined and dried over anhydrous MgSO₄. The solvent was removed using a rotary evaporator and the crude product purified by silica gel column chromatography (50% vol CH₂Cl₂ in petroleum ether). Removal of the solvent was achieved with a rotary evaporator to afford a white waxy solid (3.00 g, 4.1 mmol, 62.0%). ¹H NMR (500 MHz, CDCl₃): δ (ppm) 8.74 (d, 1H, aromatic), 8.31 (dd, 1H, aromatic), 7.75 (d, 1H, aromatic), 5.81 (m, 1H), 5.56 (s, 2H, -CH₂O), 5.01-4.85 (m, 4H), 2.44 (t, 2H), 2.04 (m, 2H), 1.66 (m, 2H), 1.38-1.25 (m, 10H). ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 173.17, 162.93, 147.77, 139.36, 138.46, 134.62, 129.52, 129.27, 126.76, 114.37, 62.61, 60.76 (t, 61.00, 60.57), 34.28, 33.87, 29.47, 29.35, 29.23, 29.06, 25.04. MS (FD) 745.2 (100%).
2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluoro-1-octyl 4-(11-trichlorosilyl-1-oxoundecyloxymethyl)-3-nitrobenzoate [3].

The alkene [2] (0.60 g, 0.81 mmol) was added to a round bottomed flask attached to a schlenk line. Air was removed and replaced with N₂. The alkene was dissolved in dry CH₂Cl₂ (2 mL) and trichlorosilane (4.0 mL) was added. A solution of hydrogen hexachloroplatinate (IV) hydrate (20 µL, 15.6 mg) in 2-propanol (0.5 mL) was injected into the flask via micro syringe. The solution was heated to reflux and allowed to react overnight. Removal of the solvent was achieved via reduced pressure on a schlenk line to afford a waxy solid. The product was used to form SAMs on glass surfaces without further purification. 

\[ ^1H \text{ NMR} (500 \text{ MHz, CDCl}_3): \delta (\text{ppm}) 8.74 (d, 1H, aromatic), 8.31 (dd, 1H, aromatic), 7.75 (d, 1H, aromatic), 5.56 (s, 2H, \text{-CH}_2\text{OOC-}), 4.89 (t, 2H, \text{-OCH}_2\text{CF}_2\text{-}), 2.44 (t, 2H), 1.66 (m, 2H), 1.56 (m, 2H), 1.29 (m, 14H). \]

\[ ^{13}C \text{ NMR} (500 \text{ MHz, CDCl}_3): \delta (\text{ppm}) 173.17, 162.93, 147.81, 138.40, 134.62, 129.52, 129.31, 126.76, 62.61, 60.76, 34.28, 31.98, 29.56, 29.45, 29.37, 29.30, 29.17, 25.03, 24.46, 22.44. \]

Microchannel Formation

All microscope slides and cover slips were first cleaned with a freshly prepared “piranha” solution (70:30 v/v conc H₂SO₄:H₂O₂) at 60 °C for 30 min, rinsed with DI H₂O and dried with a stream of clean air. Microchannels were made by affixing No. 1 glass cover slips onto a glass microscope slide (Fisher Scientific) with a UV-curable glue in such a way that the cover slips form the side walls of an H-channel. The glue was cured for 5 min with a Kinsten Exposure Unit (KVP-30, Computronic Corp LTD, Australia). The process was repeated with a larger coverslip to form the top wall of the microchannel. The resulting microchannels were approximately 190 µm deep. Plastic micropipette tips were trimmed to a length of 0.5 in and attached to the
opening of the microchannel with DURO Quick Set epoxy resin to form the inlet and outlet wells of the microchannel.

**FSAM formation and Photopatterning**

A solution is prepared by dissolving the trichlorosilyl [3] (22 mg) in toluene (8.0 g) in a scintillation vial. The solution was allowed to age for a certain period of time until 1 min immersion of a “piranha” treated coverslip resulted in a H₂O advancing contact angle of 118°. Once optimal aging has been achieved the microchannels are filled with this solution and allowed to stand for 1 min. After exposure, the microchannels are rinsed with hexane, followed by methanol and allowed to dry with a clean stream of air. If the aging solution becomes cloudy, the solution is filtered with a 0.45 µm PTFE syringe filter and the aging process is repeated until a contact angle of 118° is again achieved.

Photopatterning of the FSAM was achieved by filling the microchannels with a 0.1 M HCl solution. A photomask is placed to cover one half of the H-channel and a cover slip was placed onto the photomask to ensure that it lies flat. The UV intensity of Novacure systems (EFOS, Model N2001-A1) was preset at 5600 mW/cm², the distance between the lens and the channel was 40 mm, and the irradiation area was 20 mm in diameter. The microchannel was irradiated for 6 min. The inlet and outlet ports were also irradiated to create the hydrophilic arms of the H-channel. The microchannels were rinsed with DI H₂O, followed by methanol and dried with a stream of clean air. A sample coverslip was also irradiated in the same manner to measure the advancing contact angle. UV-irradiated FSAM decreased from 118° to 67°.
**Peptide membranes**

All peptide membranes were created in the microchannels in the same manner. Differences in reaction time varied for each peptide sequence. A basic solution of NaOH and DI water was prepared at a concentration of 16.2 mg NaOH/mL. The peptide was dissolved in this basic water solution at a concentration of 0.20 mmol peptide/mL. A solution of freshly distilled adiptyoyl chloride was dissolved in toluene at a concentration of 0.25 mmol/mL. The peptide solution was introduced into the hydrophilic arm of the H-channel. The toluene solution containing adiptyoyl chloride was then introduced to the hydrophobic arm. The solutions were allowed to react for varying times (typically 15 min). The H-channel was carefully rinsed with methanol and dried with a stream of clean air. Membranes were checked for continuity with a microscope and also checked for porosity by flowing methanol through one arm and observing that the peptide membranes contain methanol.

**Enzymatic Digestion**

Enzymatic Digestion of peptide membranes were conducted in two ways, static conditions and under fluid flow. Chymotrypsin was dissolved in pH 7.8 phosphate buffer solutions at varying concentrations (0.025-0.25 mg/mL). Under static conditions, the microchannels were filled with chymotrypsin solution, the tips covered to prevent evaporation, and placed in a 37 °C oven at varying times. The microchannels are then rinsed with DI H₂O followed by methanol and dried under a gentle stream of clean air.

The membranes were also subject to enzymatic digestion via fluid flow. The microchannels were filled with chymotrypsin solution. The hydrophobic arm was attached to a
syringe pump and the outlet of the hydrophobic arm was emptied. The syringe pump was set to a continuous cycle of infusion (4 min) and withdraw (4 min) at a flow rate of 600 µL/h.

Control experiments with buffer solution and chymotrypsin containing an inhibitor were conducted in a similar manner.

3.11 REFERENCES


